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Isolation and Characterization of a Cold-Active Xylanase Enzyme from *Flavobacterium* sp.

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Abstract. Xylan is the major component of hemicellulose, and xylan should be fully utilized to improve the efficiencies of a biobased economy. There are a variety of industrial reaction conditions in which an active xylanase enzyme would be desired. As a result, xylanase enzymes with different activity profiles are of great interest. We isolated a xylanase gene (*xyn10*) from a *Flavobacterium* sp. whose sequence suggests that it is a glycosyl hydrolase family 10 member. The enzyme has a temperature optimum of 30°C, is active at cold temperatures, and is thermolabile. The enzyme has an apparent K_m of 1.8 mg/ml and k_{cat} of 100 sec^{-1} for beechwood xylan, attacks highly branched native xylan substrates, and does not have activity against glucans.

Xylan is a complex polysaccharide that is a major component of hemicellulose [27]. The backbone primarily consists of a polymer of beta-1,4-D-linked xylose residues. In addition, there are many chemically different side branches that make the complete hydrolysis of the substrate more difficult [4, 5, 22]. The endoxylanase enzymes (EC 3.2.1.8) attack the internal beta-1,4 bonds of the xylose chain. Endoxylanases can be categorized based on the three-dimensional structure of the catalytic domains that can be deduced from the amino-acid sequence [15]. Most endoxylanases are assigned to either glycosyl hydrolase (GH) family 10 or 11. GH10 members are generally larger than those of GH11. In addition, GH11 xylanase enzymes usually have higher specific activities, but GH10 xylanases are more efficient at attacking a wider variety of substrates, including xylan decorated with various side-branching chemical moieties [7, 21].

There is great interest in obtaining different xylanases that will function at the various reaction conditions used in industry [6, 23]. Much of the research and development effort has been directed at discovering thermoactive enzymes [3, 24, 26]. The higher reaction temperatures are appealing for reasons such as sterilization, enhanced rate, and increased availability of substrate, but there is decreased process-energy efficiency. There has been much less work on cold-active xylanases. However, this class of enzymes has received increasing interest because some industrial processes require the use of lower temperatures to avoid altering or denaturing the product [9, 14]. There can also be considerable energy savings for those reactions that can be efficiently conducted at lower temperatures.

We constructed a genomic DNA library from a *Flavobacterium* sp. that was found in a local streambed and that was demonstrated to have activity against a xylan substrate. We cloned an endoxylanase gene (*xyn10*) that is a GH10 family member [15]. The enzyme hydrolyzed native xylan substrates and had no significant activity against glucan substrates. The enzyme had an optimum activity temperature of 30°C, although it had 30% activity even at 4°C. As with other cold-active enzymes, the Xyn10 enzyme is thermolabile [1, 8]. To the best of our knowledge, this is the first xylanase gene cloned from a *Flavobacterium* sp.

Materials and Methods

Bacteria strains. *Escherichia coli* strains BL21(DE3) pLysE (Novagen, Madison, WI), XL1-Blue (Stratagene, La Jolla, CA), and

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SOLR (Stratagene) were cultured in Luria-Bertani (LB) broth. Strain MSY2 is a psychrotrophic bacterium that was isolated from a soil sample taken from a creek bed in Martinez, CA, using an enrichment medium containing 0.2% monobasic potassium phosphate, 0.2% potassium nitrate, 0.0005% magnesium sulfate heptahydrate, 0.0002% calcium carbonate, 0.0001% ferric sulfate heptahydrate, and 0.1% raw corn starch (Sigma, St. Louis, MO) at 4°C.

Genomic library construction and screening. Strain MSY2 was grown in liquid culture for 48 hours at 22°C and pelleted by centrifugation. Genomic DNA was isolated from the cell pellet using the FastDNA kit (Bio101, Carlsbad, CA). The resulting genomic DNA was partially digested with *Apo*I restriction enzyme. The digested DNA was separated by electrophoreses on an agarose gel, and the 4- to 10-kb fragments were isolated using the QIAEXII Gel Extraction Kit (Qiagen, Valencia, CA). The resulting DNA was ligated to the Lambda ZAPII phage vector (Stratagene), and the ligation product was packaged into phage heads using the Gigapack Packaging Extract (Stratagene). The phage library was used to infect XL1-Blue bacteria, and the infection was mixed with top agar and poured over NZY (0.5% sodium chloride, 0.2% magnesium sulfate, 0.5% yeast extract, and 1% casein hydrolysate) agar plates. The resulting plaques were overlaid with agarose containing 0.1% RBB-xylan (4-O-methyl-D-glucurono-D-xylan-remazol brilliant blue R) (Sigma). Plaques forming clearings in the dye-labeled xylan were isolated, and individual genomic clones were obtained in SOLR bacterial cells according to the manufacturer's instructions (Stratagene).

16S rRNA analysis. Genomic DNA isolated from strain MSY2 was used as template in a polymerase chain reaction (PCR) with primers directed against the 5′ (27f) and 3′ (1525r) ends of the 16S rRNA [17]. The gene was cloned into the pCR-4Blunt vector (Invitrogen), sequenced, and compared with other 16S ribosomal RNA sequences by BLAST (basic local alignment search tool) [2].

Xylanase gene cloning. Genomic clones with xylanase activity were sequenced and analyzed for potential xylanase-encoding open reading frames using Vector NTI software (Invitrogen). The putative xylanase gene was amplified by PCR using the primers Y2-X-5 and Y2-X-3, which were designed to correspond to the 5′ and 3′ ends of the gene. *Nde*I and *Xho*I restriction enzyme recognition sites were engineered into the linker regions of Y2-X-5 and Y2-X-3 primers, respectively: Y2-X-5: CGCCATATGAAATTTATTAACCATTGTT TGCCACTAT TATC and Y2-X-3: GCGCTCGAGTTCTTTTACTTCTTTTAATTG CATAACGCTGT.

The amplified xylanase gene and the pET22b plasmid (Novagen) were digested with *Nde*I and *Xho*I, and the fragments were ligated to create the pET22-Xyn10 expression vector. The 3' end of the xylanase gene was fused in-frame to a 6X histidine-tag sequence encoded by the pET22b plasmid.

Protein expression and purification. The Xyn10 enzyme was expressed and purified from *E. coli*. The pET22-Xyn10 expression construct was transformed into the BL21(DE3)pLysE bacterial strain and grown at 30°C in LB liquid culture. When the culture density reached 0.5 (OD_{600}), isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce protein expression. After an additional 2 hours of growth, the cells were pelleted by centrifugation. The cell pellet was resuspended in 50 mM sodium phosphate (pH 7), 10 mM imidazole, 300 mM sodium chloride, and 1 mM phenyl methyl sulfonyl fluoride. The suspension was frozen in liquid nitrogen, thawed, and sonicated to lyse cells. The lysate was centrifuged for 15 minutes at 13,000 x*g*, and the supernatant was collected and passed through a 0.45-μm filter. The clarified lysate was applied to a nickel sepharose matrix (HisTrap HP column; GE Healthcare, Piscataway, NJ), and the Xyn10 enzyme was eluted with an imidazole gradient (10 mM to 500 mM) in buffer consisting of 50 mM sodium phosphate (pH 8) and 300 mM sodium chloride.

Protein activity assays. For solid-phase assays, bacterial colonies containing expression vectors were spotted onto LB agar containing 0.1% RBB-xylan and 1 mM IPTG and incubated at 37°C overnight. Active enzyme that was secreted from the bacterial colony produced clearings in the dye-labeled xylan.

Apparent kinetic parameters for the formation of reducing sugars based on mass concentrations of substrate were calculated for reactions using beechwood xylan (1 to 10 mg/ml) (pH 7) (Sigma) in 100 μl volumes at 20°C. Reactions were stopped by adding 150 μl DNSA solution (1% dinitrosalicyclic acid and 30% potassium sodium tartrate in 0.5 M sodium hydroxide) and heating the samples to 100°C for 5 minutes. Aliquots of the reaction mixture (150 μl) were measured on a 96-well plate reader. Xylose solutions were used as standards to determine concentration.

The following substrates were also tested: wheat arabinoxylan, rye arabinoxylan, carboxymethyl cellulose, Avicel, laminarin, and lichenan. Wheat and rye arabinoxylan were obtained from Megazyme (Bray, Ireland). All other substrates were purchased from Sigma. Aside from Avicel, all substrates were soluble. All liquid activity assays were conducted in 25 mM universal buffer (pH 4 to 10) (8.3 mM each of citric acid, monobasic potassium phosphate, and boric acid).

Results and Discussion

Isolation of *Flavobacterium* sp. strain MSY2. Psychrotrophic bacteria isolated from creek bed soil samples were streaked onto culture plates containing RBB-xylan. A yellow colony that produced a clearing on this substrate was isolated, cultured, and designated strain MSY2 (NRRL B-41356). Strain MSY2 was found to be a Gram-negative rod. Analysis of the strain by the Biolog Gram Negative Identification System (Biolog, Hayward, CA), an automated system that measures utilization of 96 sole carbon sources, classified strain MSY2 as *Flavobacterium johnsoniae* [11]. However, the database for *Flavobacteria* in the Biolog system was somewhat limited, so a more definitive identification was made by 16S rRNA analysis. Genomic DNA was prepared from the MSY2 strain, and 16S rRNA sequence was isolated from the DNA and analyzed (GenBank DQ196345) [17]. BLAST analysis of the 16S rRNA indicated that the strain was a *Flavobacterium* sp. Sequence analysis showed that the sequence was 99% identical (1452 of 1462 nucleotides) to the 16S rRNA of the Antarctic psychrotroph, *F. hibernum* (GenBank L39067.1). Thus, MSY2 is a *Flavobacterium* sp., and based on 16S rRNA analysis, it is possible that it may be *F. hibernum*.

Previously, there was a report that *F. frigidarium*, isolated from Antarctic marine sediment, also has xylanase activity [16]. However, the *F. frigidarium* xylanase was not biochemically characterized, so the physicochemical characteristics of the enzyme activity are currently unknown.

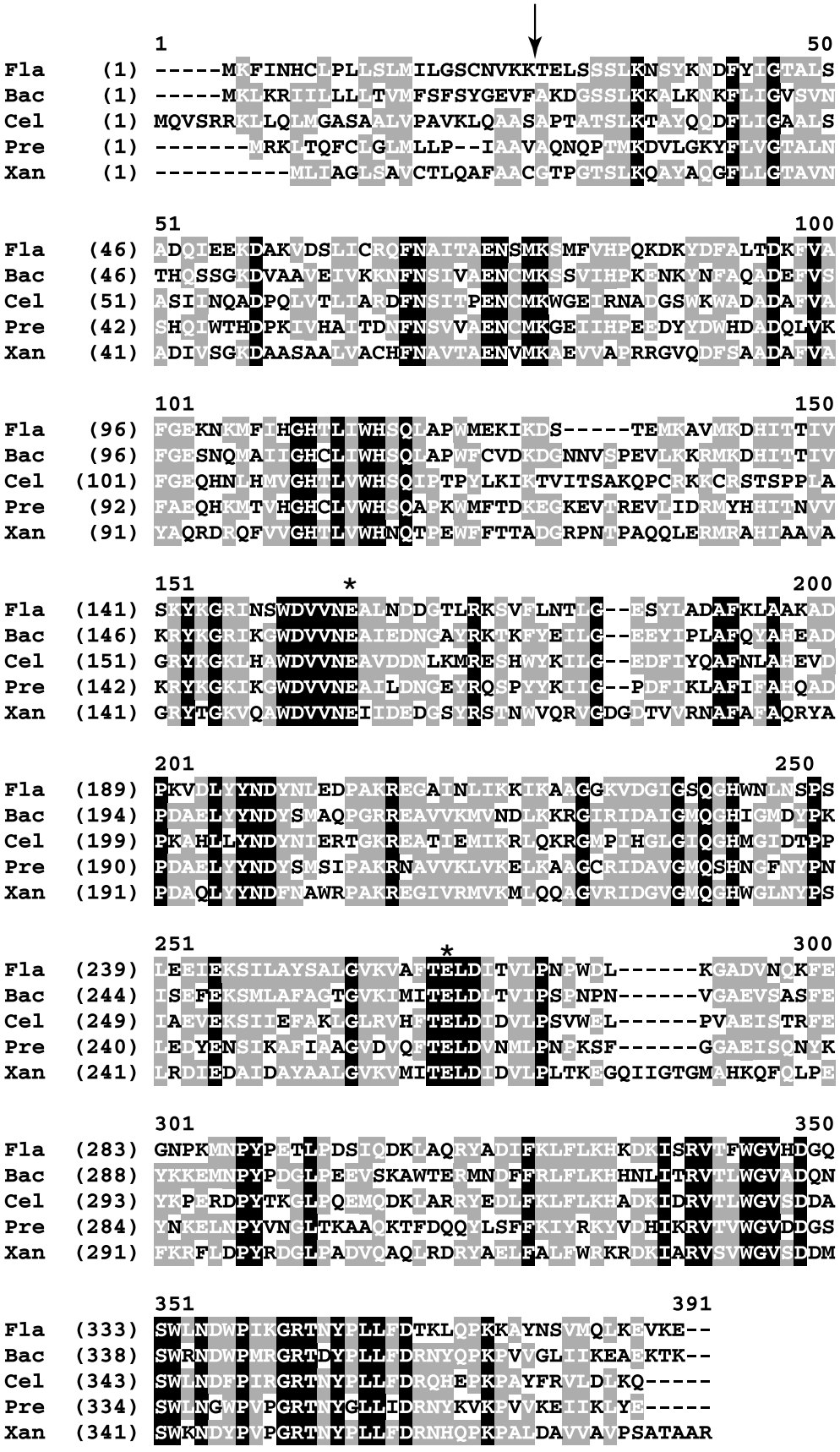

Fig. 1. *Flavobacterium* sp. xylanase protein sequence and comparison with other Xyn10 enzymes. Arrow marks the predicted cleavage site of the signal peptide sequence. Stars above residues indicate the conserved catalytic amino acids. Fla, *Flavobacterium* sp. (this study); Bac, *Bacteroides ovatus* [28]; Cel, *Cellvibrio mixtus* [12]; Pre, *Prevotella ruminicola* [13]; and Xan, *Xanthomonas axonopodis* [10].

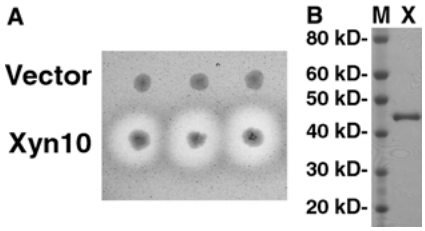

Fig. 2. Xylanase expression construct and purified protein. (A) Xylanase expression construct transformed into BL21(DE3)pLysE spotted onto LB containing 0.1% RBB-xylan. (B) Purified xylanase enzyme on SDS-PAGE gel. RBB, 4-O-methyl-D-glucurono-D-xylan-remazol brilliant blue R; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Cloning and purification of *xyn10*. Genomic DNA isolated from the *Flavobacterium* sp. strain MSY2 was used to produce a phage genomic DNA library. Those clones that produced clearings in the xylan substrate overlay were purified, and the DNA was isolated in SOLR bacterial cells. The plasmids were sequenced, and a putative open reading frame for the *xyn10* gene was discovered (GenBank DQ059337). The gene was predicted to encode a 42-kDa enzyme with a pI of 8.6 (Fig. 1). There was a signal sequence at the N-terminus of the protein that was calculated to be cleaved after Lys23 [20]. The *xyn10* gene was homologous to other cloned *xyn10* genes and contained the highly conserved acidic residues essential for catalysis [18, 19, 25].

To demonstrate activity of the encoded enzyme, the *xyn10* gene was subcloned into an expression vector and transformed into bacteria, and the resulting clones were spotted onto solid media containing RBB-xylan (Fig. 2A). Clearings around the colonies indicated that the Xyn10 enzyme was active. Purified enzyme was obtained from bacterial cultures that were induced, lysed, clarified, and processed on an affinity column (Fig. 2B). Soluble Xyn10 enzyme, 20 mg/L, was expressed in the liquid culture, and protein was purified to apparent homogeneity with a yield of 37%.

Biochemical characterization of Xyn10. The pH and temperature optimums were determined using beechwood xylan as substrate. Xyn10 was active across a broad pH range with peak activity at pH 6 to 7 (Fig. 3A). The Xyn10 enzyme maintained 40% and 60% activity at pH 5 and pH 9, respectively. A temperature profile of Xyn10 demonstrated that the enzyme had an optimal activity at 30°C (Fig. 3B). The enzyme was active at very low temperatures and retained 30% activity at 5°C. In addition, Xyn10 was very heat labile. The half-life of the enzyme was 40 minutes at 45°C and only 30 seconds at 50°C (data not shown).

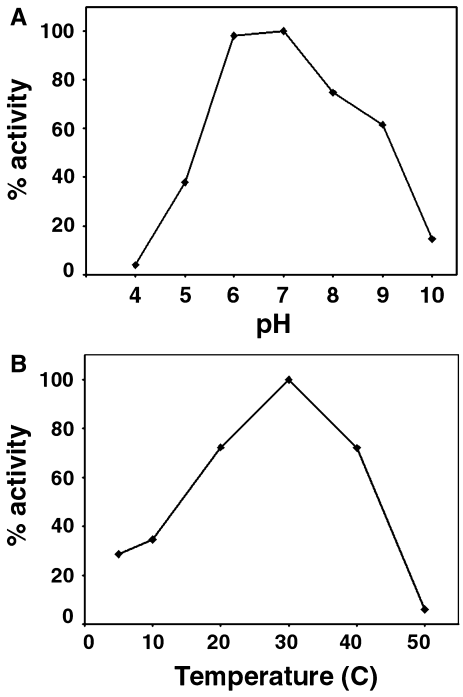

Fig. 3. pH and temperature activity profile of Xyn10 enzyme. (A) Xyn10 enzyme was incubated with beechwood xylan at various pH at 30°C. (B) Xyn10 was incubated with beechwood xylan at various temperatures at pH 7.

When tested against other carbohydrate substrates, Xyn10 had greatest activity on beechwood xylan (data not shown). Relative to the beechwood xylan, the enzyme had 50% and 40% activity against the arabinoxylans from wheat and rye, respectively. Xyn10 had almost no activity against substrates with a glucose backbone (carboxymethyl cellulose, Avicel, laminarin, and lichenin). Against beechwood xylan (pH 7) at 20°C, Xyn10 had an apparent K_m of 1.8 mg/ml, V_{max} of 142 μmole/min/mg, and k_{cat} of 100 sec^{-1}. Another cold-active xylanase has been cloned and characterized from *Pseudoalteromonas haloplanktis*, an Antarctic bacterium [8]. Compared with the *Flavobacterium* sp. Xyn10, the *P. haloplanktis* enzyme has a higher apparent k_{cat} (1,250 sec^{-1}) but also a higher apparent K_m (28 mg/ml) against birchwood xylan at 25°C.

In summary, we isolated a cold-active xylanase enzyme that does not have significant cellulase activity from a *Flavobacterium* sp. This enzyme represents a target for directed evolution experiments that will further increase its utility in commercial processes that rely on low reaction temperatures.

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